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TECHNICAL REPORT 8406

SPECTRODENSITOMETRIC QUANTITATIVE DETERMINATION OF TRICHOTHECENES IN WATER: APPLICATION TO T-2 TOXIN AND T-2 TETRAOL

AD-A149 404

ELIZABETH P. BURROWS
EVELYN H. MCNAMEE
CHARLOTTE M. TROUT

U S ARMY MEDICAL BIOENGINEERING RESEARCH & DEVELOPMENT LABORATORY
Fort Detrick
Frederick, Maryland 21701

OCTOBER 1984

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER TECHNICAL REPORT 8406	2. GOVT ACCESSION NO. AD-A149404	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) SPECTRODENSITOMETRIC QUANTITATIVE DETERMINATION OF TRICHOTHECENES IN WATER: APPLICATION TO T-2 TOXIN AND T-2 TETRAOL		5. TYPE OF REPORT & PERIOD COVERED Technical Report June 1983-September 1984
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) ELIZABETH P. BURROWS EVELYN H. MCNAMEE CHARLOTTE M. TROUT		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS US Army Medical Bioengineering Research and Development Laboratory, ATTN: SGRD-UBG Fort Detrick, Frederick, MD 21701-5010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A 3M161102BS12/AE/428
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, MD 21701-5012		12. REPORT DATE October 1984
		13. NUMBER OF PAGES 19
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Include

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Densitometry,	T-2 Tetraol,
Field Water and Sanitation,	T-2 Toxin, and
Quantitative Analysis	~~Thin Layer~~ Chromatography
Solid Phase Extraction	Trichothecenes,

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Trichothecene toxins were isolated from dilute aqueous solutions (1-4 mg/L) by adsorption on solid phase extraction (SPE) columns followed by elution with methanol. After thin layer chromatographic (TLC) separation, quantitative determinations were made by a spectrodensitometric method specific for trichothecenes. The extraction/quantitation procedure worked equally well for the relatively non-polar toxin, T-2, and the most polar trichothecene, T-2 tetraol. Recoveries were 95-100 percent for T-2 and 80-95 percent for tetraol. Originator supplied keywords:

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ACKNOWLEDGMENT

We thank Ms. Theresa M. Trybus for assistance with the initial densitometric determinations.

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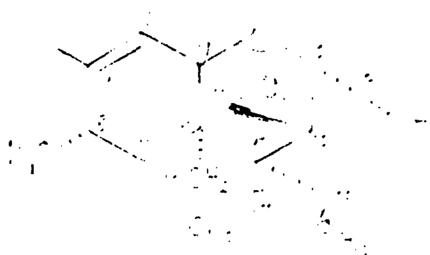
INTRODUCTION AND OBJECTIVE

T-2 [4,15-diacetoxy-3-hydroxy-8-(3-methybutyryloxy)-12,13-epoxytrichothec-9-ene] (**1a**) is an important member of the 12,13-epoxytrichothecene class of mycotoxins, produced in nat   by a number of genera of fungi and recognized increasingly since the late 1960's as responsible for toxic effects in livestock, poultry, and humans from consumption of contaminated food grains.[1] More recently, since 1981, the issue of possible use of these toxins as chemical/biological warfare agents has been hotly debated in the public and scientific press.[2,3] While numerous analytical procedures for their extraction, separation, and quantitative determination in food-related matrices have been published, their determination in trace amounts in water has not been addressed. We sought to develop a sensitive method specific for 12,13-epoxytrichothecenes in the presence of other trace organics which could be applied to field sampling. The method selected was thin layer chromatographic (TLC) separation followed by spectrodensitometric quantitation.[4] For isolation and concentration of the toxins from water, a solid phase extraction procedure was devised which was found to be as effective as conventional liquid/liquid extraction for the most polar trichothecene, T-2 tetraol (**1b**), as well as for T-2.



1a $R_1 = \overset{O}{\overset{\|}{C}}CH_2CH(CH_3)_2,\ R_2 = \overset{O}{\overset{\|}{C}}CH_3$
1b $R_1 = R_2 = H$

MATERIALS AND METHODS

EQUIPMENT

A Kontes Model 800 scanning densitometer (Kontes Scientific Instruments, Vineland, NJ, 08360) equipped with a Hewlett Packard 3390A integrator was operated in transmission mode with a 615 nm phosphor disk. A Camag automatic TLC Sampler I (Applied Analytical Industries, Wilmington, NC, 18405) was used in the solid phase extraction studies.

CHEMICALS

T-2 toxin and T-2 tetraol were purchased in 25 mg and 5 mg quantities, respectively, from Sigma Chemical Company. Tetraethylenepentamine (TEPA) and 4-(p-nitrobenzyl)pyridine (NBP) were Fisher reagent grade and were used without further purification.

PREPARATION OF STANDARDS

A stock solution of T-2 was prepared by dissolving 25 mg of T-2 in 1.0 mL of methanol. From it, solutions to deliver 800 ng, 600 ng, 400 ng, 200 ng, and 100 ng per 2 μL were prepared by serial dilution. T-2 tetraol standards were prepared in a similar manner except that the stock solution consisted of 5 mg in 1.0 mL of methanol.

For T-2, fresh standards were made up daily; for tetraol, at least once a week, and more frequently if acceptable standard curves could not be obtained.

LIQUID/LIQUID EXTRACTION

Five milliliters of aqueous solutions of T-2 (1 and 4 mg/L) were each extracted with two 1 mL portions of methylene chloride, and the extracts were concentrated under nitrogen (N_2) to approximately 100-200 μL, transferred quantitatively to micro conical vials, and blown to dryness. The residues were reconstituted in 50 μL of methanol and capped. Four standards (100 ng, 200 ng, 600 ng, 800 ng) and two samples of low and two of high concentrations were applied manually, 2 cm apart, to each E. Merck silica gel 60 plate (0.25 mm thick), using a 2 μL Drummond microcap pipette.

SOLID PHASE EXTRACTIONS

A Baker-10 solid phase extraction (SPE)[5a] system equipped with 3 mL octadecyl (C_{18}) reverse phase columns and 15 mL column reservoirs was used. The columns were conditioned as recommended by the manufacturer,[5b] by aspiration at 15 psi of one reservoir volume of methanol through them followed by 2 reservoir volumes of water, with care not to let the column go dry.

For each toxin, triplicate 5 mL portions of solutions of high concentration (4 mg/L) and of low concentration (1 mg/L) were placed into reservoirs and were aspirated through the columns at 5 psi. The columns each were washed with 3 mL of water, aspirated 30 min at 18 psi and blown dry 15 min under N_2. The adsorbed toxins were eluted by slow aspiration (3-5 psi, flow rate 1-2 mL/min) of three successive 0.5 mL portions of methanol into 2 mL vials. The eluates were taken to dryness under N_2 and the residues were reconstituted in 50 μL of methanol, transferred to micro conical vials and capped. The Camag Automatic Sampler I was used for application of the solutions to E. Merck silica gel 60 high performance (HP) TLC plates (0.20 mm thick). Operating parameters are given in Appendix A. Four standards (100 ng, 200 ng, 600 ng, 800 ng) and three samples of low and three of high concentrations were applied to each of three plates.

QUANTITATIVE TLC DETERMINATIONS

The plates, precut to 10 x 20 cm, were prewashed in chloroform/methanol (9.5:0.5), air-dried, heated 30 min in a 110°C oven, and stored in a desiccator until ready for use. The developing tank was lined with saturation pads and was allowed to equilibrate 20 min in the appropriate chloroform/methanol solvent system (9.5:0.5 for T-2 and 4:1 for tetraol were chosen, to give R_F values of approximately 0.4 in each case) before development of the plate. The chromogenic reagents employed for visualization and quantitation of the toxins were a 3 percent solution of NBP and a 10 percent solution of TEPA in carbon tetrachloride/chloroform (3:2). The former, stored under refrigeration, was stable for 1 week; it was necessary to prepare the latter fresh daily. The plates were dipped in a Whatman stainless steel tank filled with NBP solution, air dried, and placed in a 150°C oven for 30 min. (After removal of plates from the oven, it was possible to store the plates overnight in a desiccator before continuing the procedure; the remaining steps had to be carried out without interruption.) After cooling, the plates were dipped in the TEPA solution, and the toxins appeared as blue spots on a white background. The plates were covered with clear glass plates and, after 5 min to allow the background to clear, were scanned, adsorbent side down. Absorbance of the spots was recorded as peak areas on the Hewlett Packard 3390 A integrator under the conditions specified in Appendix B. Thirty minutes scanning time was required for four complete scans of standards and extracted samples across each plate.

RESULTS AND DISCUSSION

Certain difficulties inherent in the method soon became apparent. Most troublesome were non-uniformities or imperfections in the plate which may be errors of manufacture or lines due to dipping. The double beam densitometer scans background and sample and subtracts background. An imperfection in background space produces an artificially low sample reading, whereas an imperfection in sample space produces an artificially high sample reading. Fading of the spots was also a problem, especially for tetraol and at low concentrations.

The following procedure was devised to minimize the deleterious effect of these problems. The average of three or four scans of each standard spot was plotted against its concentration in ng/2 µL to produce the standard curve for that plate. The average of the scans for each sample spot was then compared to the standard curve and the concentration of the spot determined. Scan values that were far outside acceptable confidence levels or that showed marked fading were discarded. Similarly, any concentrations well outside acceptable confidence levels were discarded. The mean, relative standard deviation and percent recovery were determined for all acceptable samples on all plates from a given day. If the standard curve on a plate did not have a sufficiently high correlation (at least 0.98) or if all recoveries on that plate were too low (<50%) or too high (>110%), the plate was discarded. Typical standard curves for T-2 and tetraol are shown in Figures 1 and 2, respectively.

Table 1 summarizes the best results obtained in three consecutive days for liquid/liquid extractions of T-2. Standard deviations were generally substantially greater with manual application of samples and use of ordinary silica gel plates. The results from SPE of T-2 and tetraol, using HPTLC plates and the Camag autosampler, are summarized in Tables 2 and 3, respectively. In both cases the first 3 days were consecutive. Table 3 also documents the frequency of data discards or rejects found necessary to obtain the data summarized (column 3). At least two good plates and 2/3 of the samples spotted were used in each day's data.

Comparison of the data for T-2 (Tables 1 and 2) shows SPE to be as efficient as conventional liquid/liquid extraction; recoveries in both cases were complete. On comparison of the tables, it should be emphasized that the determinations listed in Table 1 were the most precise of a number of 3 day sets; typically standard deviations were substantially greater, with no significant differences in averaged percent recoveries. Tables 2 and 3, on the other hand, were more typical data obtained with allowance for discarding bad plates as described previously. Precision of the SPE determinations was significantly increased by both the use of HPTLC plates and the autosampler for sample application. The autosampler is more precise than a highly experienced analyst, and the HPTLC plates provide a more uniform surface producing less random background noise.

Comparison of the data for T-2 and tetraol shows that the slopes of standard curves for the latter are approximately 2/3 to 3/4 those of the T-2 standard curves. Thus tetraol exhibits a lower sensitivity to visualization by the chromogenic reagents. This is not unexpected, as differences in sensitivity of these reagents had been observed earlier for some other trichothecenes.[4] This lower sensitivity and increased uncertainty due to fading is a probable explanation for the larger standard deviations of tetraol relative to T-2. While recoveries of the former appeared somewhat lower, still they fell within a predictable, reproducible range: 80 to 99 percent for the low concentration and 85 to 100 percent for the high concentration.

In this study, concentrations of 1 and 4 mg/L were chosen for convenience, but the method may be applicable to lower concentrations by passing larger volumes of solution through the SPE column. It should be noted, however, that a limiting volume for complete retention of a solute exists, dependent on the capacity factor (k') of the solute,[6] and would have to be determined empirically. For T-2 standards, spots in the range 100 to 800 ng gave highest correlations; <100 ng amounts faded too fast, and above 800 ng flattening of the curve became a problem.[7] While tetraol was studied in the same range, it is probable that a somewhat higher range of standards would be optimal for this compound. Inspection of the two typical standard curves (Figures 1 and 2) shows both y intercepts > 0. This was commonly the case, even with curves giving the highest correlations, and may be related to plate background.

In conclusion, despite its limitations, the method has shown an acceptable degree of accuracy and precision for 2 trichothecenes of opposite extremes in polarity. Recoveries from aqueous solutions by SPE are high and predictable, and slopes and scan values for the standards are consistently predictable. This consistency allows the analyst to detect and reject a "bad" plate very readily.

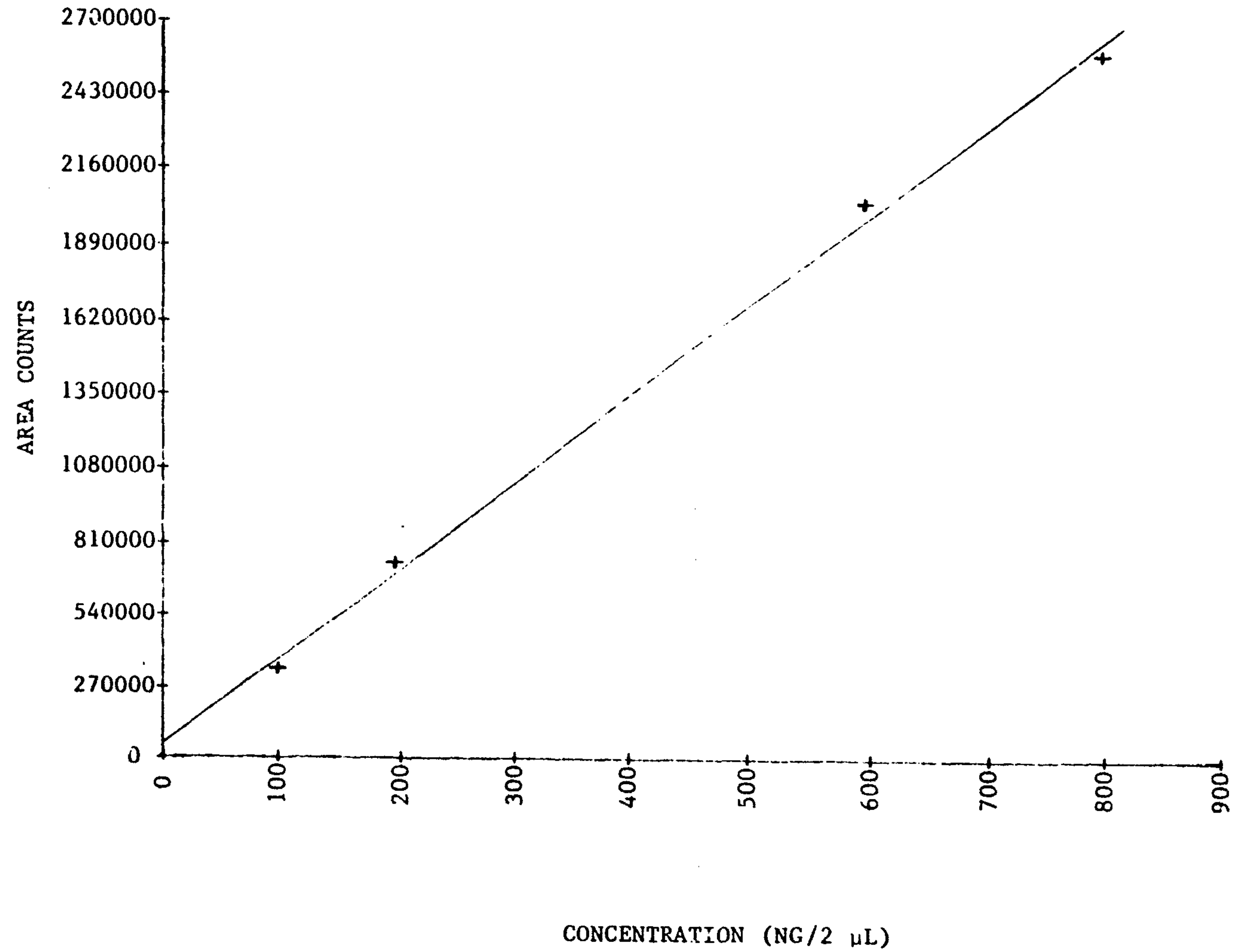


Figure 1. Standard Curve for T-2 Toxin, $R^2 = 0.9977$.

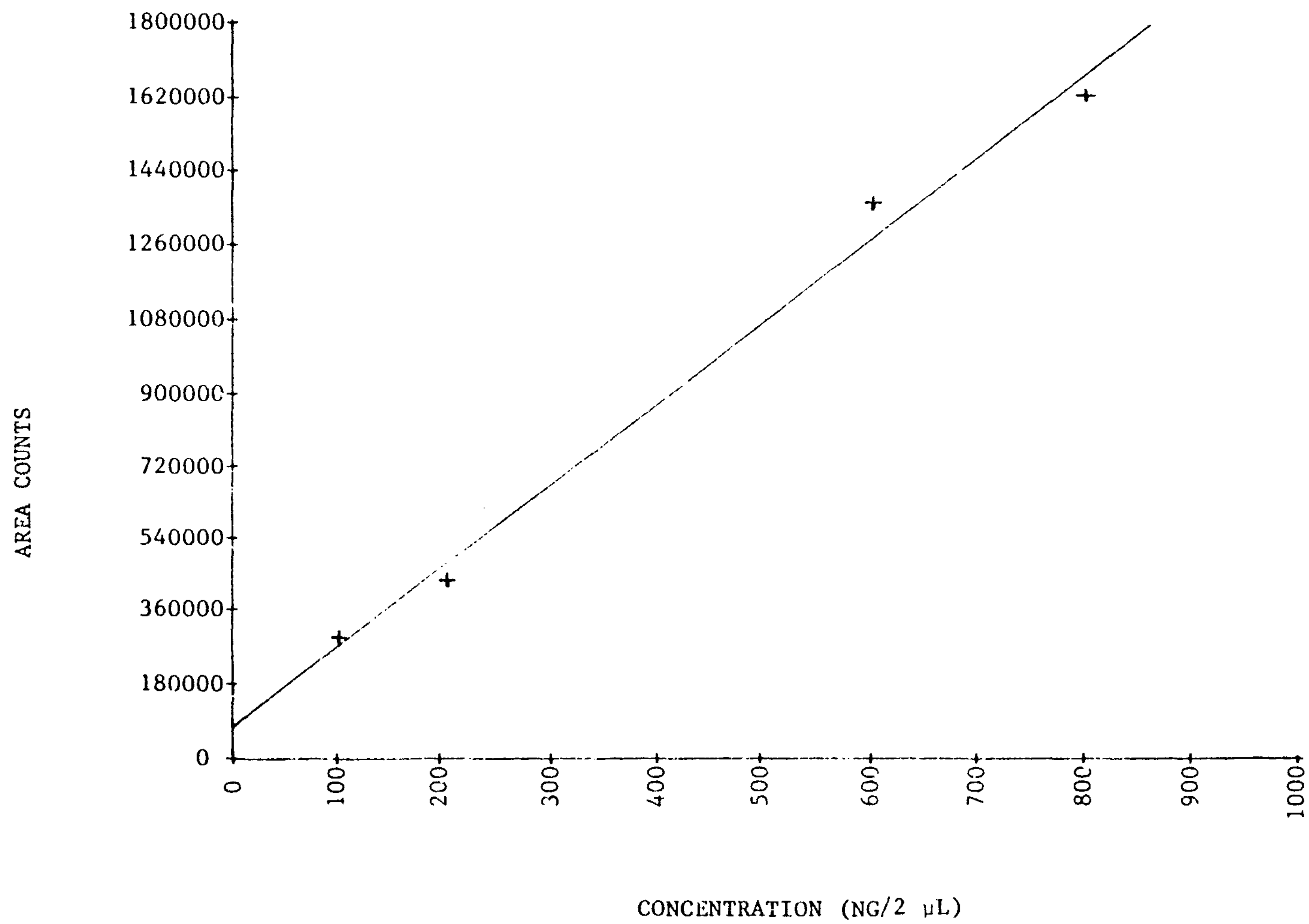


Figure 2. Standard Curve for T-2 Tetraol, $R^2 = 0.9956$.

TABLE 1. ANALYSES OF T-2 TOXIN FROM LIQUID/LIQUID EXTRACTIONS

Day	Mean (ng)	Standard Deviation	Relative Standard Deviation	% Recovery
		Low Concentration		
1	218.2	10.01	4.59	109.1
2	221.4	7.99	3.57	110.7
3	201.7	10.71	5.31	100.9
Average	213.7	9.54	4.49	106.9
		High Concentration		
1	824.8	26.2	3.18	103.1
2	806.3	18.7	2.31	100.8
3	805.1	18.8	2.34	106.9
Average	812.1	21.2	2.61	101.5

Standard Curve Data

Day	Average Slope	Average Correlation
1	3454	0.9988
2	3200	0.9986
3	4032	0.9999
Average	3562	0.9991

TABLE 2. ANALYSES OF T-2 TOXIN FROM SOLID PHASE EXTRACTIONS

Day	Mean (ng)	Standard Deviation	Relative Standard Deviation	% Recovery
		Low Concentration		
1	190.2	0.21	0.11	95.1
2	203.4	6.32	3.11	101.7
3	207.7	5.26	2.53	104.0
4	177.3	2.33	1.31	88.7
5	210.9	7.35	3.49	105.5
6	216.8	7.77	3.58	104.5
7	200.1	6.17	3.08	100.0
Average	200.9	5.06	2.46	99.9
		High Concentration		
1	785.1	3.88	0.49	98.1
2	784.2	7.40	0.94	98.0
3	826.8	4.19	0.16	103.4
4	784.3	15.4	1.96	98.1
5	766.4	16.7	2.18	95.8
6	789.9	4.60	0.58	98.3
7	757.8	16.4	2.16	94.7
Average	785.0	9.80	1.21	98.1

Standard Curve Data

Day	Average Slope	Average Correlation
1	2878	0.9942
2	3168	0.9971
3	2909	0.9990
4	3209	0.9979
5	2661	0.9997
6	3413	0.9995
7	2485	0.9939
Average	2960	0.9973

TABLE 3. ANALYSES OF 1-2 TETRAOL FROM SOLID PHASE EXTRACTIONS

Day	Mean (ng)	Number of Samples Used/ Possible	Standard Deviation	Relative Standard Deviation	% Recovery
		Low Concentration			
1	175.3	5/6	31.2	17.8	87.7
2	197.1	7/9	21.4	10.9	98.6
3	163.7	7/9	18.1	11.0	81.8
4	162.0	5/6	31.3	19.3	81.0
5	187.5	4/6	36.5	19.5	93.8
6	159.8	6/6	28.1	17.6	79.9
Average	174.2		27.8	16.0	87.1
		High Concentration			
1	755.1	5/6	86.6	11.5	94.4
2	831.3	5/9	89.4	10.8	103.9
3	738.8	9/9	50.8	6.9	92.4
4	723.8	5/6	65.0	9.0	90.5
5	843.9	5/6	42.5	5.3	105.5
6	680.4	5/6	68.1	10.0	85.1
Average	762.2		67.1	8.9	95.3

Standard Curve Data

Day	Average Slope	Number of Plates	Correlation Range
1	2162	2/3	0.9892-0.9956
2	2059	3/3	0.9907-0.9993
3	2057	3/3	0.9876-0.9943
4	2137	2/3	0.9942-0.9968
5	2324	2/3	0.9983-0.9994
6	2225	2/3	0.9893-0.9956
Average	2160	Total Range	0.9876-0.9994

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APPENDIX A

OPERATING PARAMETERS FOR CAMAG AUTOMATIC SAMPLER I

Dialog:

Plate width = 200 mm
No. vials = 10
Plate edge space = 25-30 mm
Sample space = 17 mm
Deliv speed = 50 nL/sec
Sample volume = 2,000 nL

Service Dialog:

Capillary vol. = 24,000 nL (24 μL)
No. air bubbles = 1
Vol. air bubbles = 100 nL
Surplus vol. = 100 nl
Rinsing vol. =
TOT Vol ? (1/0) 1 = yes
Vol. predosage = 200 nL
Filling time = 500 nL/sec

APPENDIX B

OPERATING PARAMETERS FOR KONTES MODEL 800 SCANNING DENSITOMETER AND HEWLETT PACKARD 3390 INTEGRATOR

(1) Kontes Model 800
Scan mode = B-A
Scan Rate cm/min = 1-0
Attenuator = 512
Output normal

(2) HP 3390A Integrator
Zero = 10
Att 2 = 8
CH+Sp = 2
PKWD = 0.12
THRSH = 4
AR Reg = 15,000
Intg #2 times 0.1

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